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Simultaneous enantioselective separation of azelastine and three of its metabolites for the investigation of the enantiomeric metabolism in rats
I. Liquid chromatography–ionspray tandem mass spectrometry and electrokinetic capillary chromatography

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Dedicated to Professor Ernst Bayer, one of the pioneers of chromatography and capillary electrophoresis

Abstract

Enantioselective separation methods and the enantioselective determination of the anti-allergic drug azelastine and of three of its main phase I metabolites in a biological matrix underwent chromatographic and electrophoretic investigations. An enantioselective assay of a coupling of HPLC using a β-cyclodextrin chiral stationary phase to ionspray tandem mass spectrometry is presented. Additionally, this assay is compared to another enantioselective assay using electrokinetic capillary chromatography with β-cyclodextrin and carboxymethyl-β-cyclodextrin in polyacrylamide-coated capillaries. For capillary electrophoresis (CE) the importance of polyacrylamide coating for the validation of this separation method is highlighted. Extracted rat plasma samples of enantioselective metabolism studies were measured by both validated assays. Differences in the pharmacokinetics and pharmacodynamics were evaluated for the main substance azelastine and its main metabolite demethylazelastine. So, a first hint about the enantioselectivity of biotransformation of azelastine in rats was seen after oral application of either enantiomer or the racemate to rats.

Keywords: Enantiomer separation; Azelastine; Demethylazelastine

1. Introduction

The chiral anti-allergic drug azelastine (AZ) was introduced to therapy as a racemate. As there was no clear difference observed between the enantiomers in pharmacological tests, possible differences in the pharmacokinetics and biotransformation of azelastine enantiomers have not yet been investigated. Simultaneous quantitative enantioselective assays of azelastine enantiomers and its main metabolite enantiomers were not available. Former achiral metabolic studies revealed the formation of demethylazelastine (DAZ)

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and also of two monohydroxylated metabolites 6-hydroxyazelastine (6-OH-AZ) and 7-hydroxy-azelastine (7-OH-AZ), respectively, as phase I metabolites in humans and rats [1]. Additionally, N-oxides of azelastine (D-20228, D-19144) are formed in rats [2] (Fig. 1). Those two AZ-N-oxides were first detected during studies of the AZ racemate with rat liver microsomes (unpublished data). Until now, only one enantioselective determination of azelastine in a biological matrix was proposed using a high-performance liquid chromatography (HPLC) column switching technique and detection by mass spectrometry (MS) [3]. Former enantioselective separations of the azelastine racemate were performed for qualitative purpose using ion chromatography [4], under aspects of chiral separation abilities of new chiral stationary phases (CSPs) [5,6] or of chiral selectors in capillary electrophoresis (CE) [7,8].

For the measurement of extracted rat plasma samples, firstly an enantioselective HPLC assay was performed using fluorescence detection. Column selection and optimisation for enantioselective separation of all compounds are described. This assay was conducted with a coupling to tandem mass spectrometry (HPLC–MS–MS) for higher selectivity and sensitivity reasons.

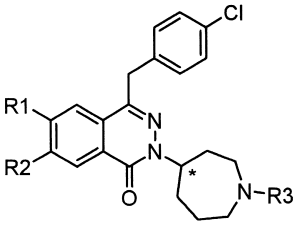
The method development in enantioselective CE with different cyclodextrins and its validation are discussed. The evident advantage of using polyacrylamide-coated capillaries in CE is pointed out. This assay is compared to the HPLC–MS–MS assay and the results from the pharmakokinetic studies are presented.

2. Experimental

2.1. Chemicals and reagents

Azelastine, its enantiomers and metabolites DAZ, 6-OH-AZ and 7-OH-AZ, all as their hydrochlorides and DAZ as hydrobromide (Fig. 1) were a kind gift of ASTA Medica (Frankfurt/Main, Germany), as well as the internal standards flezelastine hydrochloride (only for achiral CE experiments) and D-17795, an azelastine analogue (Fig. 2), rat plasma and rat plasma samples. The animal study was conducted by ASTA Medica.

2-Propanol, ethanol, acetonitrile and *n*-hexane of HPLC grade and methanol were purchased from Baker (Deventer, The Netherlands), and diethylamine and triethanolamine from Fluka (Buchs, Swit-



substance	*abbreviation*	*R1*	*R2*	*R3*
azelastine	AZ	H	H	CH_3
demethylazelastine	DAZ	H	H	H
6-hydroxy-azelastine	6-OH-AZ	OH	H	CH_3
7-hydroxy-azelastine	7-OH-AZ	H	OH	CH_3
azelastine-N-oxide	AZ-N-oxide	H	H	O, CH_3

Fig. 1. Chemical structures of azelastine and metabolites AZ, DAZ, 6-OH-AZ and 7-OH-AZ, AZ-N-oxide.

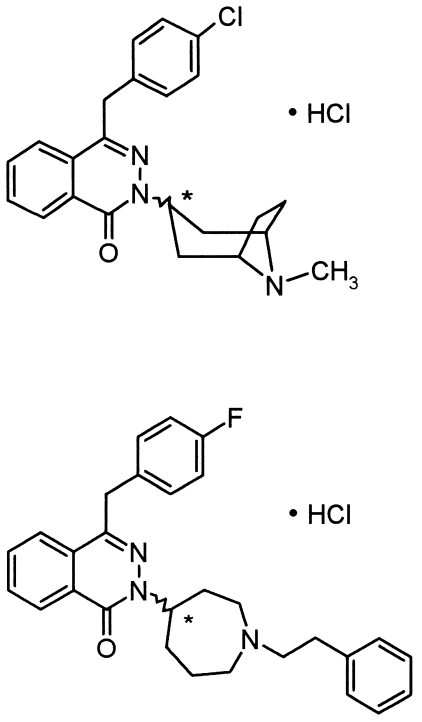


Fig. 2. Chemical structures of the azelastine analogue D-17795 (above) and flezelastine hydrochloride (below).

zerland), ammonium acetate and solutions of sodium hydroxide or hydrochloric acid and phosphoric acid were from Merck (Darmstadt, Germany). Water was prepared by a Milli-Q plus system from Millipore (Bedford, MA, USA).

Chiral CE investigations and also achiral method development were done with fused-silica capillaries from Polymicro Technologies [Phoenix, AZ, USA, 47 cm (40 cm to the detector)×50 μm I.D.×365 μm O.D.]. Detailed experimental conditions are given in the corresponding figures. For achiral investigations an aqueous buffer of 85% orthophosphoric acid was prepared and adjusted with triethanolamine to an appropriate pH adding methanol. For chiral separations, this buffer contained β-cyclodextrin and carboxymethyl-β-cyclodextrin from Wacker (Burghausen, Germany). Capillaries were coated with polyacrylamide [9] using methanol, acrylamide, *N*,*N*,*N′*,*N′*-tetramethylethylenediamine, sodium persulfate, *tris*(hydroxymethyl)aminomethane, boric acid and magnesium sodium ethylendiaminetetraacetate from Merck (Darmstadt, Germany), and γ-methylacryloyloxypropyl-trimethoxysilane was supplied by Fluka.

2.2. Design of the enantioselective metabolism study

To male Wistar rats one single dose of 50 mg/kg either azelastine racemate or one enantiomer was applied orally (by oral gavage). Plasma samples were collected at 0 and 0.5, 1, 3 and 6 h after application, whereas one time point corresponds to one sample of one animal. Due to the fact that each sample had a volume of at least 3 ml, each sample represents one animal. The heparinized plasma samples were immediately deep frozen (about −28 °C) and stored for at maximum 2 months. This study was performed twice, firstly for the investigation of the extracted samples by HPLC–MS–MS and secondly for using the electrokinetic capillary chromatography (EKC) assay. Ethical reasons did not allow to sacrifice more animals for these two studies.

2.3. Sample preparation

Stock solutions and internal standard solution (diluted in hydrochloric acid) were added prior to extraction of the 3-ml rat plasma samples and incubated for 30 min at room temperature. Those samples were made alkaline by adding 150 μl of 1 mol/l sodium hydroxide. Extraction was performed once with 12.5 ml diethyl ether and twice (7.5 and 12.5 ml) with a mixture of *n*-hexane–*n*-octanol (99:1, v/v) to extract the highest possible amounts of AZ and DAZ of that sample volume, which could be derived from one rat. All three organic extracts were combined and re-extracted into 300 μl of 0.01 mol/l hydrochloric acid. An aliquot of 100 μl of the aqueous extract was analysed by HPLC–MS–MS. For the EKC assay re-extraction was performed with a volume of 80 μl hydrochloric acid to gain a high concentration of the analytes. From this a 20-μl aliquot was used.

2.4. High-performance liquid chromatography

Stock solutions of the analytes were prepared with ethanol. As CSPs, a Chiralpak AD column of 250 mm×4.6 mm (10 μm material) from Daicel (Tokyo,

Japan) and a Cyclobond I 2000 of 250 mm×4.6 mm (5 μm material), obtained from Astec (Whippany, NY, USA), were used.

HPLC was performed with a Merck–Hitachi L-1000 pump (Tokyo, Japan), a Rheodyne Model 7125 from Rheodyne (Cotati, CA, USA), and a UV detector 655A or a fluorescence spectrophotometer F-1050 from Merck–Hitachi and a Merck–Hitachi D-2500 integrator (as above). For temperature conditioning of the column a Haake conditioning device with a Haake S bath (Haake Messtechnik, Karlsruhe, Germany) was employed containing ice water. For enantiomeric purity of both AZ enantiomers 50 m*M* ammonium acetate buffer, pH 4.7–acetonitrile–methanol (80:12:8, v/v) was used.

2.5. High-performance liquid chromatography–mass spectrometry

Extracts of the plasma samples were measured applying the Cyclobond I 2000 analytical column with a corresponding pre-column of 30×4.6 mm. The HPLC equipment consisted of a LiChrograph L-6200 intelligent pump from Merck–Hitachi and an autosampler Waters 717 plus from Waters (Milford, MA, USA). The column was cooled by a cryostat FT 401 (Julabo Labortechnik, Seelbach, Germany) using 2-propanol. A Sciex API 300 triple quadrupole mass spectrometer from PE Sciex (Toronto, Canada) with a pneumatically assisted electrospray ion source (TurboIon Spray) in combination with heated nitrogen as auxiliary gas (i.e., turbo gas) was employed at a temperature of +400 °C and an ion spray voltage of +4800 V (positive ion mode). A mobile phase of 45 m*M* ammonium acetate, pH 4.7–methanol–acetonitrile (70:21:9, v/v) using a flow-rate of 0.4 ml/min was applied. As post-column sheath liquid acetonitrile was added rectangular to the eluent via a stainless steel capillary using a Valco Tee-junction with a flow-rate of 0.4 ml/min. Therefore, a deactivated fused-silica capillary (100 μm) was directly connected ''post-column'' to the analytical column by the mixing-Tee and was then fed through this device while approximately reaching 4 cm into a second stainless steel capillary. The latter led directly into the TurboIon Spray interface of the mass spectrometer.

2.6. Capillary electrophoresis

All achiral and chiral developments in fused-silica capillaries were performed with an aqueous buffer of 85% orthophosphoric acid in several different concentrations and adjusted with triethanolamine to different pH values while applying normal polarity and rinsing steps with methanol, water and buffer. The CE device consisted of a Beckman System P/ACE 5510 (Beckman Coulter, Wakefield, MA, USA) with a P/ACE UV-absorbance detector using a filter of 214 nm and Beckman Gold software (version 8.1). Samples were injected hydrodynamically into the capillary with 0.5 p.s.i. nitrogen overpressure for 16 s at 25 °C, afterwards injecting water for 1 s as on-line concentration (1 p.s.i.=6894.76 Pa).

Validation was performed in coated capillaries using a 100 m*M* phosphoric acid buffer, 7 mg/ml CM-β-CD and 17 mg/ml β-CD at pH 5.0 applying −596 V/cm at 20 °C. The structure analogue of AZ, D-17795, was applicable as internal standard. Rinsing steps of methanol (3 min), 50 m*M* H_3PO_4 of pH 2.5 (5 min), water (5.5 min) and buffer (1 min) were applied.

2.7. Coating procedure of capillaries

Polyacrylamide coating of the fused-silica capillaries was performed manually with a syringe in three steps. In general the coating procedure of Hjertén [9] was followed, however it had to be standardised to assure reproducibility of the capillary coating for validation.

Firstly, the surface of the capillary was activated. A pre-rinse of 1 h with 1 mol/l sodium hydroxide and double-distilled water, respectively was performed in the Beckman CE device with 20 p.s.i. overpressure. Residues of water were blown out of the capillary with 20 p.s.i. nitrogen overpressure and eliminated in a drying chamber by heating for 120 min at 120 °C. After a brief cooling period, the ends of the capillaries were closed immediately by a laboratory film and a detection window was etched into the outer polyimide coating by heating. The following silanisation was directly started with a solution of equal volumes of γ-methylacryloyloxypropyltrimethoxysilane and methanol, remaining in each capillary for 12 h and then removed completely

by rinsing with water. The following polymerisation was performed using a 4% (m/v) acrylamide–buffer solution, to which per ml monomer solution 5 μl of *N*,*N*,*N′*,*N′*-tetramethylethylendiamine and 5 μl of a 10% (m/v) aqueous sodium persulfate solution in bidistilled water was added. This monomer–buffer solution consists of 0.1 mol/l boric acid, 0.1 mol/l *tris*(hydroxymethyl)aminomethane, and 0.002 mol/l magnesium sodium ethylendiaminetetraacetate. The monomer–activator solution was immediately placed in the capillary and remained within each capillary for 12 h at room temperature. Finally the capillary was rinsed with water. As storage solution 50 m*M* phosphoric acid, pH 2.5 was suitable.

3. Results and discussion

3.1. High-performance liquid chromatography

Method development was started using a Chiralpak AD column with a CSP of a 3,5-dimethylphenylcarbamate derivative of amylose, as these columns show a wide range in separating differently structured racemates in the normal-phase mode [10]. Applying UV detection at 230 nm, investigations resulted only in an enantioseparation of the racemate of the metabolite DAZ (R_s=0.98) while using *n*-hexane with 2-propanol and diethylamine as organic modifier. However mobile phases of *n*-hexane with ethanol only allowed an enantioseparation of the azelastine enantiomers (Fig. 3). Fluorescence detection resulted only in a detection of azelastine racemate, metabolites were nonfluorescent. While using MS with Ionspray ionisation or an atmospheric pressure chemical ionisation (APCI) interface or ion-trap mass spectrometry an ionisation was impossible.

Therefore, enantioselective chromatography was switched to another CSP using an aqueous–organic mobile phase. A β-cyclodextrin column (Cyclobond I 2000) appeared to be the most suitable for enantioseparation and coupling to MS. Method development was performed in the reversed-phase mode using fluorescence detection (excitation wavelength 297 nm, emission wavelength 360 nm). First investigations showed that higher amounts of buffer or methanol, an increase of the pH or molarity of the buffer as well as a decrease of the flow-rate contributed to a better separation of all enantiomers. Due to the influence of temperature onto the separation selectivity of cyclodextrin columns [11], low temperatures of 5 °C resulted in a better resolution. Under optimised conditions of temperature, flow-rate and amount of methanol, the enantiomers of AZ (R_s=1.09) and DAZ could be separated simultaneously. However, an enantioselective resolution of both hydroxylated metabolites 6-OH-AZ and 7-OH-AZ separated from the other two compounds resulted in a weak performance. Nevertheless, enantiomeric purity of both AZ enantiomers could be measured with this method. In both enantiomers an impurity of more than 0.5% of the other enantiomer can be detected, a quantification of 1% of each enantiomer within the other is possible.

3.1.1. High-performance liquid chromatography–mass spectrometry

Chromatographic conditions used during fluorescence detection were optimised for a detection with the Sciex API 300 triple-quadrupole mass spectrometer using atmospheric pressure ionisation (API). The TurboIon Spray interface can accept high flow-rates up to 1 ml/min together with relatively high volatile buffer concentrations of up to approximately 20 mmolar without a significant loss of sensitivity (however compound dependent). This is enabled through a special ‘‘off-axis’’ sprayer geometry of the interface, which therefore turned out to be the preferred interface in biopharmaceutical analysis of thermally stable compounds. Investigations with an ion trap mass spectrometer using APCI showed a weak ionisation and a significantly higher limit of detection (LOD).

In order to achieve a high selectivity for electrospray operation in the mass spectrometer, the HPLC conditions used for fluorescence detection had to be modified to meet the requirements for electrospray HPLC–MS. The following modifications for higher sensitivity were implemented: a higher content of acetonitrile in the mobile phase at a given flow-rate resulted in a better ionisation efficiency (i.e., sensitivity) for AZ and its metabolites owing to the nature of the electrospray ionisation process. However, the higher organic content caused a decrease of enantioselectivity for all the enantiomers. Consequently, the proportion of the organic part (acetoni-

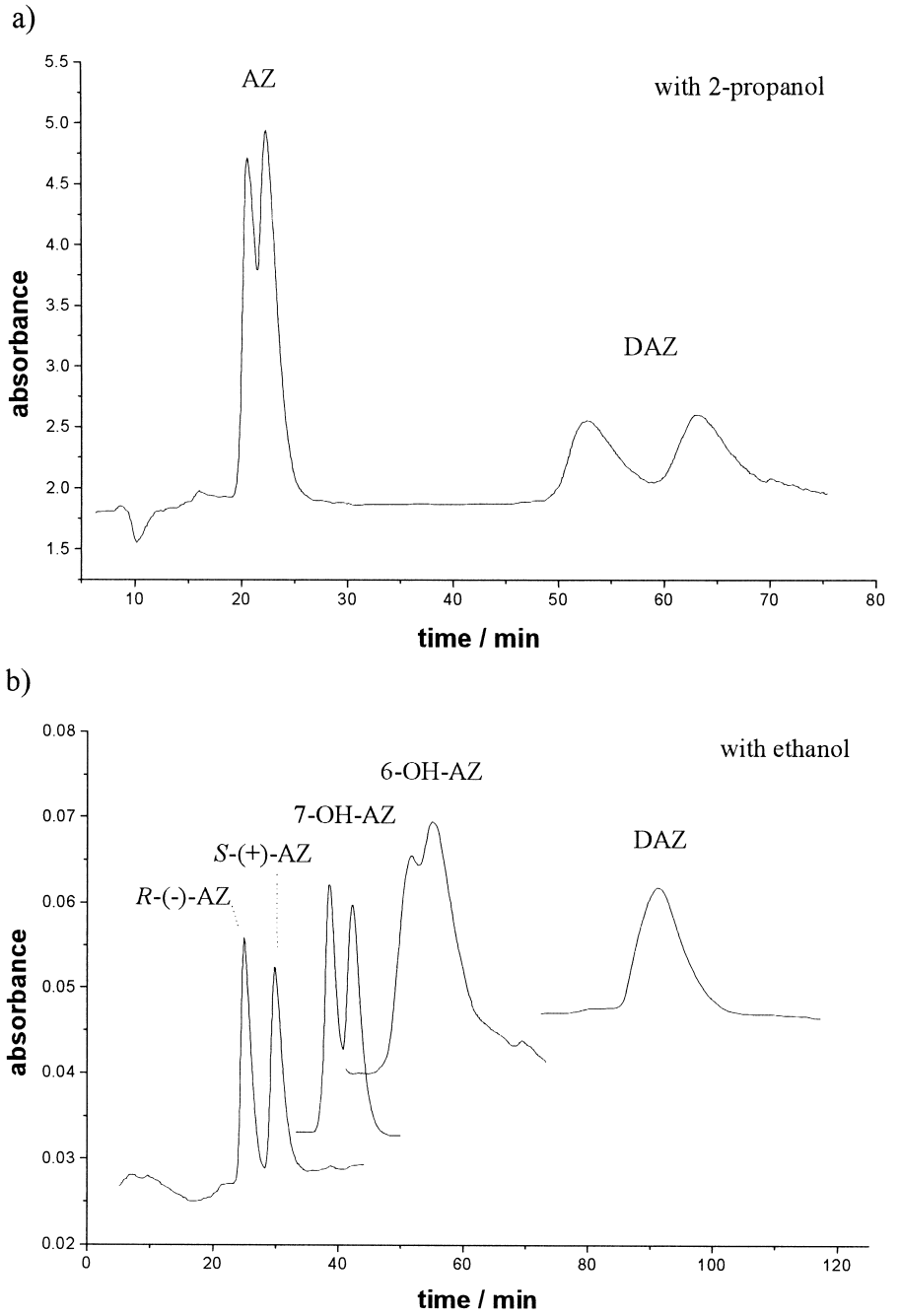


Fig. 3. HPLC separation of the enantiomers of AZ and its metabolites on a Chiralpak AD column (250×4.6 mm, 10 μm material) with a flow-rate of 1.2 ml/min and UV detection at 235 nm; (a) with *n*-hexane–2-propanol–diethylamine (95:5:0.6, v/v) and (b) with *n*-hexane–ethanol–diethylamine (97:3:0.7, v/v).

trile) in the eluent was increased via a post-column sheath-liquid flow introducing acetonitrile. Hereby, an acceptable enantioselective chromatographic separation and simultaneously sufficient sensitivity for the mass spectrometric detection was maintained. With this set up, a fourfold higher sensitivity for azelastine extracted from the rat plasma samples was reached using a sheath flow of 0.4 ml/min even taking into account the dilution effect. Hence, a compromise in terms of eluent composition (content of organic solvent and concentration of volatile buffer) was employed regarding the optimum ionisation efficiency together with an acceptable enantiomeric separation for all analytes. Co-elution of AZ and its metabolites (except that one of the two hydroxylated metabolites) was acceptable since the metabolites could easily be distinguished by their different m/z ratios (Fig. 4). The AZ-N-oxide could also be determined with a longer retention time however with the same m/z ratio as the hydroxylated metabolites.

Unfortunately no advantage of the usually very short analysis times under HPLC–MS conditions could be taken due to the demand of enantioselective separation of each substance, even while using higher temperatures of 10 °C. Nevertheless, more stable and shorter retention times resulted. A much higher selectivity of the compounds was achieved by tandem mass spectrometry in the selected reaction monitoring (SRM) mode. The SRM ion chromatograms (positive ion mode) with the corresponding m/z ratios of the product ions are presented in Fig. 4. AZ and its metabolites do not undergo any fragmentation in the TurboIon Spray ionisation on the API 300 mass spectrometer. Even at high ionspray voltages (higher than +4800 V) and high temperature (+400 °C) or at high collision energies (CAD fragmentation) no fragments were detected. This

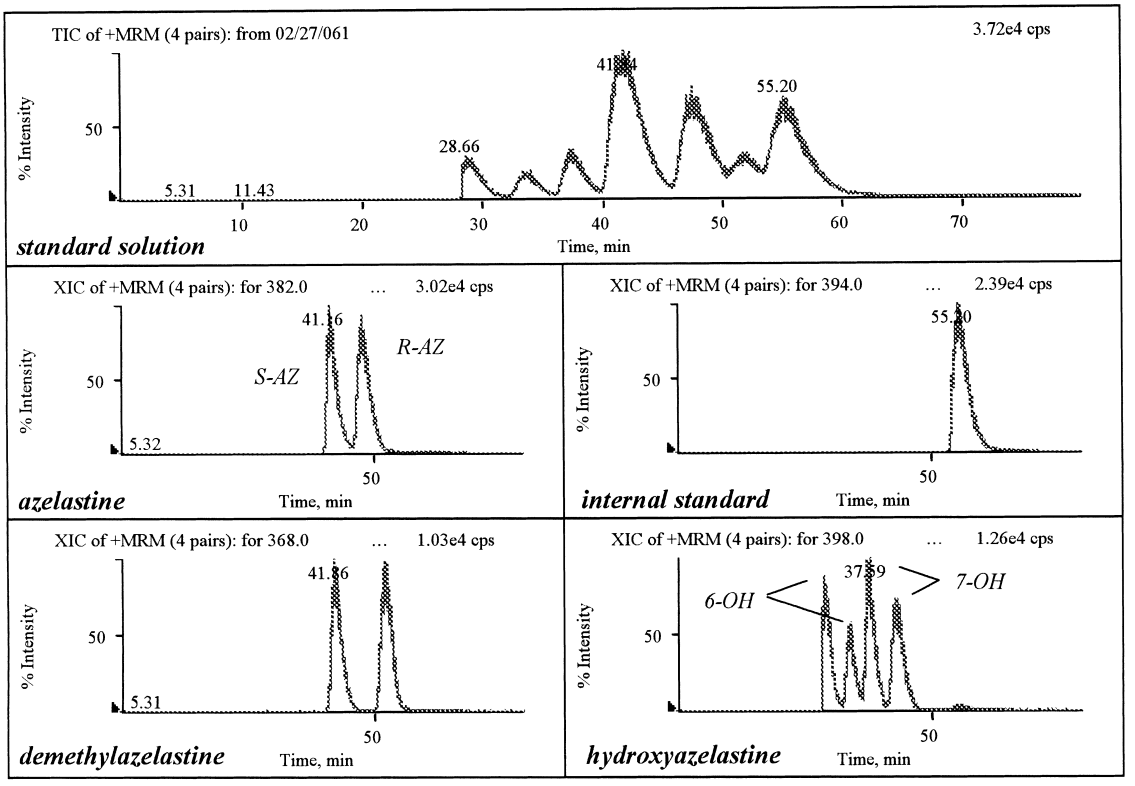


Fig. 4. HPLC–MS–MS enantioselective separation by measuring an extract of blank rat plasma spiked with a standard mixture of 160 μg/ml plasma azelastine (m/z 382), DAZ (m/z 368), 6-OH-AZ (m/z 398), 7-OH-AZ (m/z 398) racemates and D-17795 (m/z 394). Experimental conditions as described in the text.

could also be seen during ion trap mass spectrometry. Hence, azelastine and its metabolites can be taken as thermally stable and so the m/z ratios of all precursor and product ions show no difference.

3.1.2. Validation of the HPLC–MS–MS method

The validation was performed on extracted rat plasma samples investigating selectivity, precision, linearity and accuracy. The LOD and the limit of quantification (LOQ) were determined.

As internal standard the azelastine analogue D-17795 was applicable concerning retention time, mass spectrometric detection and lack of resolution into stereoisomers. The elution order is 6-OH-AZ, 7-OH-AZ before AZ-N-oxides. As a result of the threefold extraction no interference by any substance was observed within the mass chromatogram of extracted blank rat plasma. So, a suppression of the ionisation or an inter-individual influence by the matrix by hydrophilic compounds was excluded. When spiking blank plasma with the internal standard D-17795, all mass chromatograms of extracted samples showed an additional substance (quantified area to less than 1% of substance area) at the same retention time and with the same m/z ratio as the AZ-N-oxide D-19144. This substance did not occur when measuring non-extracted standard solutions with D-17795. The retention times of the hydroxylated metabolites were reproducible with relative standard deviations (RSDs) of 1.0 to 2.2% for each enantiomer. The retention time ratio of each enantiomer to the internal standard was reproducible with RSDs of 0.8 to 1.4%.

Due to the long retention times and an additional equilibration step after each run of the extracted sample, the run times resulted in 80 min. Hence, intra-day or inter-day precision studies were not performed. Daily system suitability tests were performed with a standard solution of all substances' enantiomers and with an extracted sample of blank plasma. Five concentration levels were measured for each enantiomer of each substance with two extracted samples in a working range from 125 up to 1250 ng enantiomer/ml plasma. As an LOQ (signal-to-noise ratio 10 to 1 for all substances) a concentration of 125 ng enantiomer/ml plasma was set and for the LOD a concentration of 40 ng enantiomer/ml plasma could be determined. The detection of azelastine enantiomers showed the highest sensitivity of the method compared to all other enantiomers of the metabolites. The assessment of data was done by weighted regression $1/y^2$. For the linearity of each enantiomer area to concentration plot refer to the equations in Table 1. Correlation coefficients for all enantiomers ranged from 0.997 to 0.998. The precision of each enantiomer of each substance remained below 10% (except the DAZ data in one concentration level) and the accuracy had a range of 94 to 107% (see Table 2).

In additional experiments, the same behaviour of both azelastine enantiomers during extraction from rat plasma samples could be verified. Therefore, different concentration ratios of *R*-AZ to *S*-AZ from 98:2 to 2:98 were spiked into blank rat plasma samples and extracted according to the same method as for all the other samples. A comparison of the ratio of the actual to the nominal value of the AZ enantiomers showed linearity over the whole range and could be described by nearly the same equation (for *R*-AZ $y=0.9352x+0.0495$ and for *S*-AZ $y=0.9352x+0.0152$). No enantioselective loss or adsorption, or racemisation of one enantiomer was observed during extraction or chromatography. An investigation of the solutions, which were applied to the animals, showed that the *R*-(−) enantiomer contained 2% of the *S*-(+) form, and the *S*-(+) form 1% of the *R*-(−) enantiomer.

3.1.3. HPLC–MS–MS results of the in vivo assay

The extracts of the rat plasma samples were measured in the SRM mode not only for selected

Table 1
Calibration equations for the enantiomers extracted from rat plasma measured by the HPLC–MS–MS assay

R-AZ	$y=0.761x+0.060$
S-AZ	$y=0.641x+0.060$
R-DAZ	$y=0.249x+0.002$
S-DAZ	$y=0.228x+0.011$
R-6-OH-AZ	$y=0.133x+0.005$
S-6-OH-AZ	$y=0.182x+0.012$
R-7-OH-AZ	$y=0.212x+0.005$
S-7-OH-AZ	$y=0.245x+0.006$

Ratio of the area of enantiomer to internal standard is calculated corresponding to the concentration in ng/ml plasma, using weighted linear regression $1/y^2$. Experimental conditions as described.

Table 2
Data for precision and accuracy for the HPLC–MS–MS assay

Concentration (ng/ml)	Parameter	*R*-AZ	*S*-AZ	*R*-DAZ	*S*-DAZ	*R*-6-OH-AZ	*S*-6-OH-AZ	*R*-7-OH-AZ	*S*-7-OH-AZ
1250	Mean value	0.9612	0.8172	0.3074	0.2847	0.1709	0.2306	0.2753	0.3103
	Precision (%)	1.93	0.38	6.88	1.35	2.69	8.39	9.38	7.69
	Accuracy (%)	94.78	94.49	98.04	95.90	99.82	96.00	101.80	99.47
	Residual	−0.0500	−0.0441	−0.0059	−0.0113	−0.0004	−0.0089	0.0053	−0.0020
1000	Mean value	0.8096	0.6992	0.2543	0.2463	0.1336	0.1904	0.2162	0.2467
	Precision (%)	1.59	0.50	7.03	5.62	5.30	5.96	6.68	5.19
	Accuracy (%)	99.52	101.06	102.32	104.14	98.14	99.40	100.28	99.34
	Residual	−0.0114	−0.0018	0.0033	0.0073	−0.0044	−0.0036	−0.0008	−0.0043
500	Mean value	0.4656	0.3984	0.1265	0.1279	0.0732	0.1096	0.1110	0.1328
	Precision (%)	0.57	0.34	3.74	6.04	7.76	1.59	5.33	4.34
	Accuracy (%)	106.66	105.59	100.17	102.38	102.61	106.88	99.45	103.43
	Residual	0.0251	0.0179	0.0000	0.0029	0.0017	0.0066	0.0000	0.0043
250	Mean value	0.2620	0.2286	0.0650	0.0681	0.0389	0.0595	0.0580	0.0664
	Precision (%)	0.57	2.58	13.48	14.51	9.48	4.38	10.99	5.76
	Accuracy (%)	106.27	105.48	101.70	99.86	102.07	103.74	99.19	98.72
	Residual	0.0118	0.0084	0.0008	0.0001	0.0006	0.0020	0.0000	−0.0009
125	Mean value	0.1498	0.1356	0.0327	0.0392	0.0214	0.0342	0.0322	0.0368
	Precision (%)	2.31	2.03	6.55	3.81	7.82	5.66	5.11	8.42
	Accuracy (%)	94.59	94.86	99.75	100.03	99.08	96.38	101.09	100.54
	Residual	−0.0053	−0.0045	−0.0004	−0.0003	−0.0002	−0.0006	0.0007	0.0002

Concentration of each enantiomer in ng/ml plasma after extraction is given in correlation to the corresponding mean value of the area ratio of each enantiomer to the internal standard.

m/z ratios of AZ, DAZ, OH-AZ and I.S. but also for two possibly occurring metabolites: demethylhydroxylated azelastine (m/z 384) and a metabolite with an opened azepine moiety (m/z 414). None of these substances could be detected in any mass chromatogram of the extracted samples. All detected substances were clearly identified by their ratio of retention time to the internal standard and also by spiking experiments with the corresponding racemate or AZ enantiomer. A full scan of the extracted samples was also applied. However, due to its lack of sensitivity no masses could be detected.

The measurements gave evidence that concentration and stereochemistry of the metabolites differ after application of each AZ enantiomer and also after application of the AZ racemate (Fig. 5). For each sampling time higher concentrations of the demethylated metabolite were detected than of the parent substance AZ. As the concentration of the hydroxylated metabolite 6-OH-AZ was often below the LOQ and 7-OH-AZ was detected at its detection limit, one can only speculate on their formation.

After a single application of *S*-(+)-AZ, extremely low concentrations of *S*-(+)-azelastine and also of its stereochemically corresponding metabolites were found at 0.5 and 1.0 h (Fig. 5), whereas after the application of *R*-(−)-AZ a maximum concentration of *R*-AZ and *R*-DAZ can be observed after 0.5 h. This means that *S*-(+)-azelastine is either excreted very rapidly as metabolites or may be metabolized rapidly via another unknown metabolic route. After application of the racemate, which was given in the same dose as both enantiomers, the stereoselective assay showed plasma concentration levels of the *S*-(+)-AZ and its stereochemically corresponding metabolites only in a very low concentration compared to higher concentrations of the *R*-(−)-AZ and its corresponding metabolite *R*-DAZ. However, the concentration of unmetabolized *R*-(−)-AZ was lower than half of that after a single application of *R*-(−)-

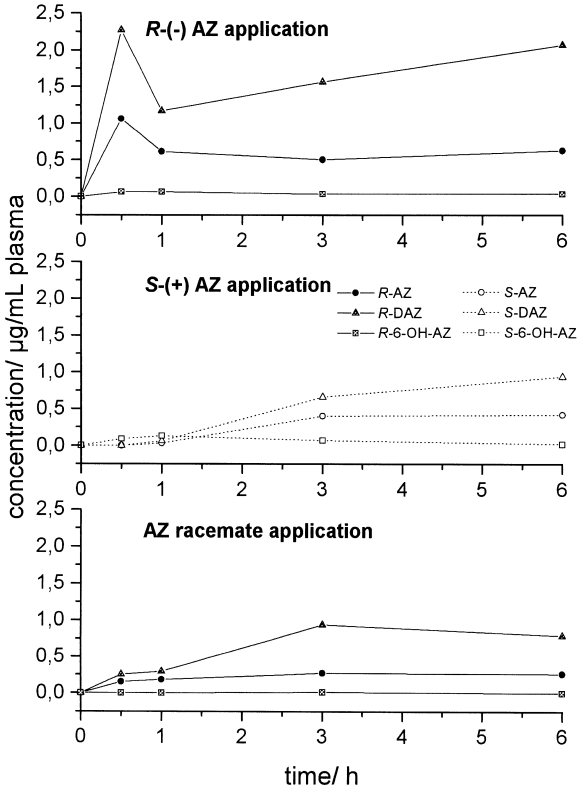


Fig. 5. Concentration–time plot of enantiomers in rat plasma samples after oral application of the single AZ enantiomer or the AZ racemate to the rats, measured by LC–MS–MS assay.

AZ. This means that a 10–50-fold excess of the *R* enantiomer compared to the *S* enantiomer was found after the application of AZ racemate to rats.

3.2. Capillary electrophoresis

Until now, no simultaneous CE separation has been described for azelastine and its metabolites. Therefore, in the first step the strategy was to gain information about the mobility of the substances by performing an achiral CE separation. On this basis an enantioselective separation was developed using the same buffer by adding cyclodextrins (CDs), which are frequently used as chiral additives for enantioseparations in CE [12]. Especially derivatives of native CDs show a very good performance in analytical measurements of basic substances [13].

3.2.1. Achiral separations

An achiral separation was developed in bare fused-silica capillaries using a buffer of phosphoric acid. The influence of the pH itself, the kind of base used to adjust the pH, the buffer concentration and the addition of methanol were investigated.

Adding triethanolamine as base to the acid buffer instead of triethylamine or 0.1 mol/l sodium hydroxide showed a decrease and acceptable modification of the electroosmotic flow (EOF). While using normal polarity and a pH value of pH 6.5 a good differentiation of the substances migrating faster than the EOF was achieved. The separations were more effective when adding methanol to sharpen the peaks due to its influence onto the interaction of the analyte with the CD cavity [14]. As internal standard the phthalazinone derivative flezelastine could be used (see Fig. 6).

3.2.2. Enantioselective investigations in uncoated fused-silica capillaries

The aim was to resolve all racemates of the metabolites as well as the azelastine racemate. Different kinds of CDs were applied to the achiral separation system. Separation conditions were optimised concerning the pH in the acidic range, the

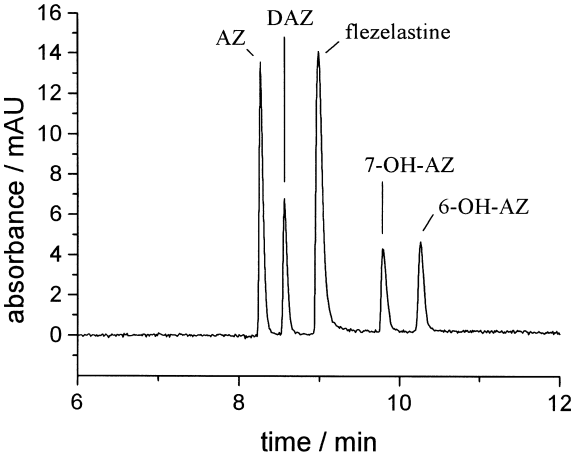


Fig. 6. Achiral CE separation of a standard mixture of the enantiomers with internal standard flezelastine in fused-silica capillaries using UV detection at 214 nm. Buffer: H_3PO_4 50 m*M*, pH 6.5 adjusted with triethanolamine, 3% methanol. Field strength: +532 V/cm. Hydrodynamic injection for 5 s at 0.5 p.s.i. overpressure.

buffer and methanol concentration. The basis of the enantioselectivity of those separations is the difference in the complex binding constants of each enantiomer of a substance to the CD [15] resulting in different mobilities of the free and complex forms of each enantiomers CD [16].

Using the native β-cyclodextrin only DAZ and 6-OH-AZ could be separated into enantiomers whereas the other two racemates remained unseparated. Also no resolution was achieved with α-CD, γ-CD, hydroxypropyl-β-CD and methyl-β-CD. The separation capabilities of the CDs always behaved in a similar manner for the analytes AZ and both OH-AZ and in contrast to DAZ, which possessed a different chiral recognition by the CDs. This is the result of its lack of N-methyl substitution and became an important fact for the migration order of DAZ enantiomers which was found for the extracted rat plasma samples.

To improve the enantioseparation of the N-methylated substances the anionic carboxymethyl-β-CD (CM-β-CD) and succinyl-β-CD in the above mentioned buffer were tested at pH 6.5. Applying normal polarity all analytes and the EOF moved towards the cathode whereas the anionic CDs migrated towards the anode. The positively charged analytes interact with both CDs in a different manner. Two racemates, AZ and 7-OH-AZ, could be separated by the CM-β-CD, however Succ-β-CD only resolved the DAZ racemate. To enable a simultaneous separation of all four phthalazinone derivatives a binary CD system of a neutral (β-CD) and an anionic CD (CM-β-CD or Succ-β-CD) was chosen to take advantage of additive or synergistic effects of the interaction between CD and analytes. Those binary CD systems are used to gain a better enantioselectivity resulting from an opposite chiral recognition by the ionic and neutral CD [16] and different effects onto the migration of the analytes [17].

The most successful enantioselective separation was obtained combining CM-β-CD (14 mg/ml buffer) with β-CD (16 mg/ml) at a slow EOF (pH 5.25) while adding methanol. Very good resolution values and number of theoretical plates were determined (see Table 3). The first migrating enantiomers showed a number of theoretical plates between 220 000 and 420 000, and all second migrating enantiomers between 110 000 and 200 000. Migration orders were determined as *R*-AZ after *S*-AZ and both faster than the metabolites' enantiomers (Fig. 7). However, a validation of this separation system remained impossible as will be described below.

3.2.3. Electrokinetic capillary chromatography

Due to the unsuccessful trial of validation in fused-silica capillaries they were coated with polyacrylamide. The EOF is suppressed completely under these conditions, and reversed polarity has to be applied. This constellation of EKC without an EOF leads to a complete opposite profile of migration order in comparison to capillary electrophoresis (CE) [18]. An investigation of the extracted rat plasma

Table 3
Performance of CE and EKC separation of the enantiomers in uncoated and coated fused-silica capillaries: resolution according to migration order (experimental conditions as in Figs. 7 and 8, respectively)

Enantiomer	Resolution (R_s)	
	Uncoated fused-silica capillary	Coated fused-silica capillary
S-AZ *R*-AZ	2.3	2.5
R-DAZ *S*-DAZ	3.6	3.2
S-6-OH-AZ *R*-6-OH-AZ	2.6	2.9
S-7-OH-AZ *R*-7-OH-AZ	5.8	2.3

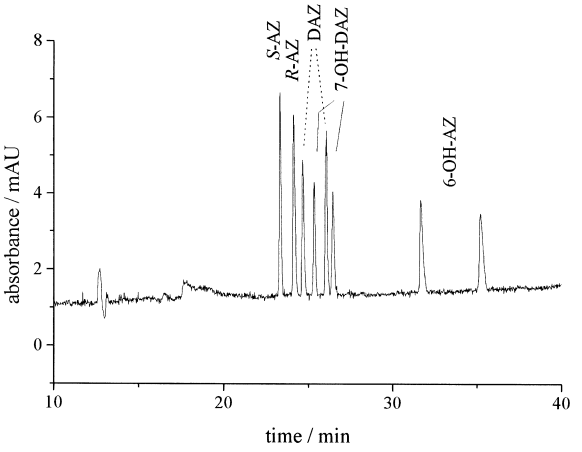


Fig. 7. Chiral CE separation of a standard mixture of the enantiomers in an uncoated fused-silica capillary using UV detection at 214 nm. Buffer: H_3PO_4 100 m*M*, pH 5.25 adjusted with triethanolamine, 3% methanol, 14 mg/ml CM-β-CD and 16 mg/ml β-CD. Field strength: +426 V/cm. Hydrodynamic injection for 8 s at 0.5 p.s.i. overpressure.

samples with the CD-mediated CE and additionally with the EKC method showed these opposite migration orders for the AZ and OH-AZ enantiomers but the same migration order for the DAZ enantiomers. This fact was verified by measuring the same sample of extracted rat plasma with both methods.

Only slight adjustments of the buffer composition and of the separation conditions were necessary to improve the chemical selective separation of the substances in the coated capillaries. The enantioselective separation still remained very good, even though the resolution was decreased (see Table 3) The first migrating enantiomers showed a number of theoretical plates between 24 000 and 46 000, and all the second migrating enantiomers between 18 000 and 35 000.

The buffer composition had to be optimised concerning CM-β-CD and β-CD concentrations pH and applied voltage.

3.3. Validation of the CD-EKC method

In uncoated fused-silica capillaries the organic modifier methanol showed a tremendous influence on migration times. A lack of reproducibility of migration times for all enantiomers arises from considerable changes in the surface of the capillary and therefore in the EOF [19–21].

To overcome all those difficulties a coating with polyacrylamide was applied and rinsing conditions optimised. The assay could then be validated according to ICH guidelines [22,23]. An entire rinsing method of each about seven capillary volumes with methanol followed by 50 m*M* phosphoric acid, pH 2.5, water and additionally separation buffer still remained necessary even while measuring standard solutions. When measuring extracted rat plasma samples, the rinsing steps had to be elongated and a rinsing with water regularly after three runs and additionally after each calibration series was performed. Hereby, a very good lifetime of about 150 runs of aqueous solutions per coated capillary could be reached (each run time about 35 min) [24]. The relative standard deviation of the relative migration times of each enantiomer to the I.S. (40 μg/ml) remained over 40 runs in a range of 0.63–0.99% for all enantiomers when measuring solutions prior to extraction. After extraction of plasma samples spiked with standard solutions the relative migration times remained reproducible over 35 runs in a range of 1.3 to 3.6%.

This assay is specific for all enantiomers of AZ and its metabolites. As internal standard the AZ analogue D-17795 was applicable for the HPLC–MS–MS assay, especially because of its lack of discrimination into enantiomers. Extracted blank plasma showed also no interferences by endogenous substances. The migration order of the AZ enantiomers was determined as *R*-(−)-AZ before *S*-(+)-AZ (see Fig. 8). Both AZ-N-oxides (each as four diastereomeric forms) could also be resolved into two peaks. The migration of both N-oxides D-20228 and D-19144 differs completely each from another (see Fig. 9). However, the relative and absolute configuration of the *RS*/*SR*- or *RR*/*SS* forms of those racemates were not investigated. In general, all substances with the N-substitution of the azepine moiety showed again another interaction of the analyte to the CD than the demethylated substance or the I.S. as a tropanyl derivative.

Measuring five concentration levels (each $n=5$) of standard solutions of AZ and its metabolites' racemates in 0.01 mol/l hydrochloric acid for a working range of 30–120 μg enantiomer per ml, precision

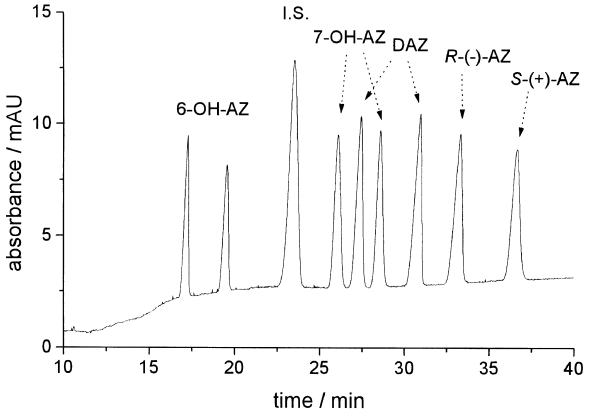


Fig. 8. Chiral EKC separation of a standard mixture of the enantiomers with internal standard D-17795 in a coated capillary using UV detection at 214 nm. Buffer: H_3PO_4 100 m*M*, pH 5.0 adjusted with triethanolamine, without methanol, with 7 mg/ml CM-β-CD and 17 mg/ml β-CD. Field strength: −596 V/cm. Hydrodynamic injection for 16 s adding 1 s water applying 0.5 p.s.i. overpressure.

and accuracy could be proved prior to extraction. Precision ranged between 3.9 and 13%. Linear regression, described by correlation coefficients, was between 0.983 and 0.998 (see Table 4). The LOD was determined as 10 μg/ml enantiomer using a signal-to-noise ratio of 3 to 1. Intra-day precision and also inter-day precision were not especially performed or calculated due to the long migration and rinsing times. However, the data show a very good performance of precision over the complete analysis time of the testing series. The reproducibility of the coated capillaries each to another ($n=5$) was verified before.

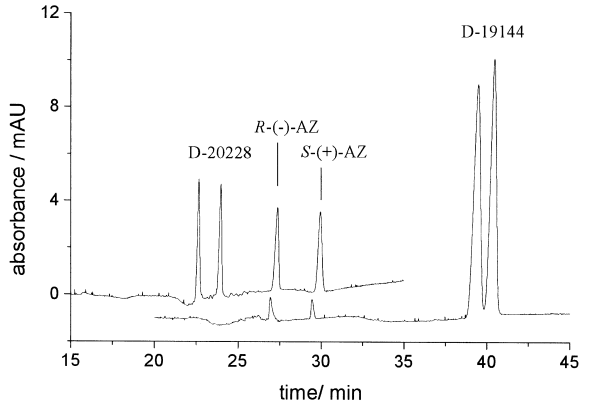


Fig. 9. Stereochemical separation of the four diastereomers of both AZ-N-oxides. Experimental conditions as in Fig. 8.

Table 4
Calibration equations for the enantiomers before and after extraction measured by the EKC assay

Enantiomer	Before extraction	After extraction
R-AZ	$y=0.0068x-0.0529$	$y=0.2912x-0.0236$
S-AZ	$y=0.0056x-0.0484$	$y=0.2079x+0.0164$
R-DAZ	$y=0.0072x-0.0288$	$y=0.2994x-0.0489$
S-DAZ	$y=0.0076x-0.0409$	$y=0.3055x+0.0081$
R-6-OH-AZ	$y=0.0079x-0.0270$	$y=0.0695x-0.0047$
S-6-OH-AZ	$y=0.0068x-0.0457$	$y=0.1196x-0.0131$
R-7-OH-AZ	$y=0.0075x-0.0363$	$y=0.2108x-0.0074$
S-7-OH-AZ	$y=0.0064x-0.0371$	$y=0.1930x-0.0077$

Ratio of the area of enantiomer to internal standard is calculated corresponding to the concentration in μg/ml, using linear regression.

After extraction of spiked plasma samples in a range of expected plasma concentrations precision and accuracy were determined for six concentration levels (each $n=5$, range from 0.27 to 17.04 μg/ml plasma) and calibration equations could be calculated for a working range of 0.53–4.27 μg enantiomer per ml plasma. The limit of detection can be given as 0.3 μg enantiomer per ml plasma. For the enantiomers of each hydroxylated metabolite and the *R*-DAZ a working range of 0.53–4.27 μg/ml was given, and for the AZ enantiomers a working range of 1.07–4.27 μg/ml was given. As the compounds' concentration in the extracted plasma samples were very low, the emphasis was laid onto the accuracy of the lower concentration levels. The lower concentrations for AZ were only evaluated to get rough information about the formation of metabolites and the biotransformation of azelastine. Precision, accuracy and residuals are given in Table 5. However, the precision values for the two upper concentration levels (8.53 and 17.07 μg/ml) are the best ones of the assay for all enantiomers (3.3 to 19.8%). Intra-day precision and also inter-day precision were not especially performed or calculated due to the long migration and rinsing times. The equations of linear

Table 5
Data for precision and accuracy for the EKC assay after extraction from rat plasma

Concentration (μg/ml)	Parameter	*R*-AZ	*S*-AZ	*S*-DAZ	*R*-DAZ	*R*-6-OH	*S*-6-OH	*R*-7-OH	*S*-7-OH
0.53	Mean value	0.0819	0.0536	0.1596	0.1235	0.0460	0.0545	0.1075	0.0904
	Precision (%)	39.0	46.3	21.2	16.6	19.0	18.0	14.3	19.5
	Accuracy (%)	67.9	42.3	93.0	107.9	136.8	106.0	102.1	95.2
	Residual	−0.0500	−0.0738	−0.0116	0.0125	0.0136	0.0037	0.0023	−0.0050
1.07	Mean value	0.365	0.325	0.360	0.295	0.056	0.118	0.232	0.204
	Precision (%)	20.5	13.4	7.5	13.1	10.3	19.5	20.1	12.6
	Accuracy (%)	125.0	136.4	107.9	107.8	82.3	102.8	106.5	102.8
	Residual	0.0779	0.0868	0.0259	0.0244	−0.0135	0.0035	0.0145	0.0058
3.20	Mean value	0.849	0.679	0.949	0.790	0.209	0.345	0.615	0.610
	Precision (%)	13.5	11.8	16.7	14.3	16.6	15.4	11.0	7.3
	Accuracy (%)	93.7	99.5	96.2	87.6	96.2	93.7	92.3	100.0
	Residual	−0.0592	−0.0027	−0.0367	−0.1192	−0.0087	−0.0246	−0.0522	0.0001
4.27	Mean value	1.250	0.893	1.334	1.310	0.300	0.514	0.927	0.815
	Precision (%)	15.5	17.9	8.5	16.5	19.3	17.5	16.4	19.8
	Accuracy (%)	102.5	98.8	101.7	106.4	102.7	103.3	103.9	99.9
	Residual	0.0310	−0.0105	0.0223	0.0814	0.0081	0.0168	0.0349	−0.0008

Concentration of each enantiomer in μg/ml plasma is given in correlation to the corresponding mean value of the area ratio of each enantiomer to the internal standard.

regression for all enantiomers were calculated in the more narrow range of the concentration levels from 0.53 up to 4.27 μg enantiomer per ml (see Table 4). Correlation coefficients range from 0.982 to 0.999. So, there was the possibility to gain rough information about the plasma levels for all substances of the study including azelastine.

Concerning possible differences between the enantiomers of each substance no enantioselective losses or adsorptions could be observed over all concentration levels. This assay shows a low rate of recovery (nominal concentration 120 μg enantiomer/ml) for the extraction of the hydroxylated metabolites (*R*-/*S*-6-OH-AZ 24.9/48.0 μg/ml and *R*-/*S*-7-OH-AZ 72.5/83.7 μg/ml) due to the hydrophobic extraction solvent, whereas *R*-/*S*-AZ (110.9/107.4 μg/ml) and *S*-/*R*-DAZ (108.8/94.5 μg/ml) are extracted to a higher extent. During the method development the robustness of the EKC assay had been proved to tolerate slight changes in separation conditions as temperature, pH value, buffer concentration, concentration of β-CD and degree of substitution of the anionic CD derivative. As a daily system suitability test, a standard solution with all racemates of AZ, DAZ and the OH-AZs was tested.

3.4. EKC results of the in vivo assay

Rat plasma samples from a second identical enantioselective metabolism study were investigated after liquid–liquid extraction. All detected substances were identified by their ratio of retention time to the internal standard and also by spiking experiments with the corresponding racemate or AZ enantiomer. As mentioned above the migration order of the DAZ enantiomers is the opposite of the other three racemates [25]. This was verified by additional experiments using buffers containing just the anionic CD and by comparison of all results of this study.

The EKC assay confirms the results of the HPLC–MS–MS assay because after the application of the *S*-(+)-AZ in comparison to the *R*-(−)-AZ or the AZ racemate concentration and stereochemistry of the metabolites show comparable results, even though each time point reflects one animal. Again, the single application of *S*-(+)-AZ results in extremely low concentrations of *S*-(+)-azelastine and of its stereochemical corresponding metabolites (Fig. 10). Higher plasma levels of *R*-(−)-AZ and *R*-(−)-DAZ are found after the application of *R*-(−)-AZ and also after application of the racemate (Figs. 10 and 11).

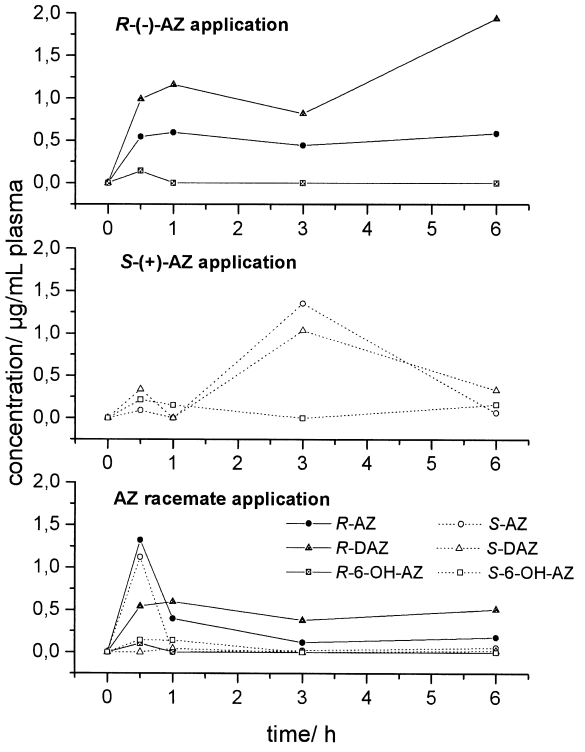


Fig. 10. Concentration–time plot of enantiomers in rat plasma samples after oral application of the single AZ enantiomer or the AZ racemate to the rats, measured by EKC assay.

Due to the lower sensitivity of the CE–UV assay compared to HPLC–MS–MS, the plasma levels are only high enough after application of the *R*-(−)-AZ to give a clear result that higher concentrations of the demethylated metabolite compared to AZ were detected. As the concentrations of both hydroxylated metabolites OH-AZ were found below the limit of quantification after extraction, as a fact of their lower recovery, it is again only possible to speculate on their formation. The OH-Azs have been more closely examined due to former animal studies showing their formation.

4. Conclusion

The enantioselective separation of four different basic azepine analytes was investigated by HPLC using an amylose tris(3,5-dimethylphenylcarbamate) and a β-cyclodextrin as chiral stationary phases. A simultaneous enantioselective determination of azelastine and its three metabolites was successful after transferring the separation method to tandem mass spectrometry. So, for the first time an analytical method was available to study enantioselective differences in the plasma concentrations and the phar-

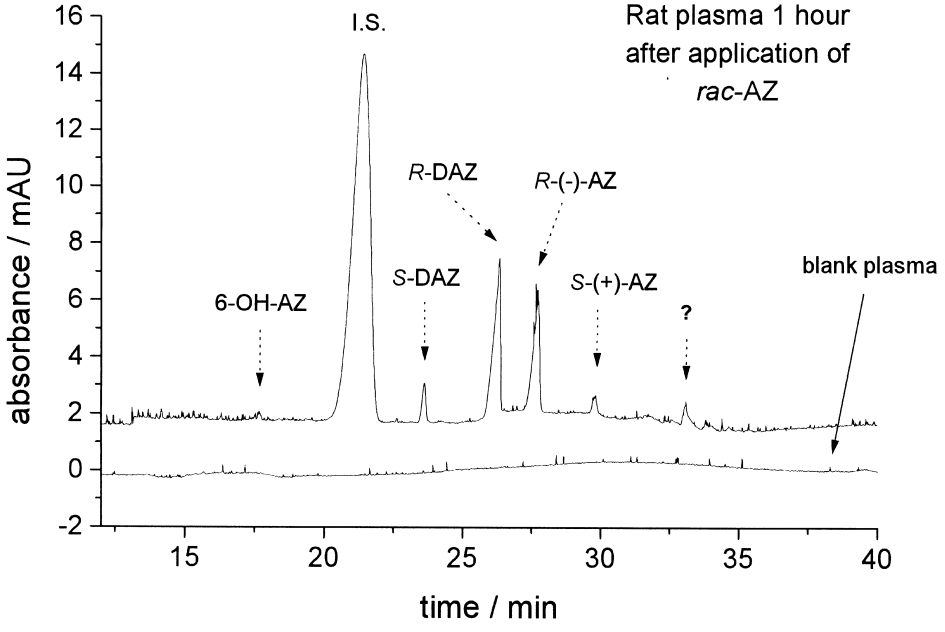


Fig. 11. Electropherogram of the EKC assay of an extracted plasma sample sampled 1 h after oral application of the AZ racemate.

macokinetic behaviour of the AZ enantiomers metabolism in rats. *S*-(+)-AZ seems to be metabolized completely different compared to *R*-(−)-AZ.

During CD-mediated CE experiments the negative influence of the interaction with the surface of the fused-silica capillary became obvious, however it could be completely suppressed by a polyacrylamide coating of the capillary. This CD-mediated electrokinetic capillary chromatography method together with an entire rinsing procedure let to reproducible migration times and ensured validation.

The results of the former HPLC–MS–MS method were confirmed by the second study and gave the same hints about the differences in the plasma concentrations and the pharmacokinetic behaviour of the AZ enantiomers. Comparing both assays each contributes to the investigation of the enantioselective metabolism of AZ and shows advantages which can be related to general aspects of the corresponding separation method. On the one hand tandem mass spectrometry with a good selectivity and lower LOD for the analytes, and on the other hand EKC with a very effective enantioseparation and low sample volumes and material costs.

Analytical HPLC and CE methods are now available for the investigation of the enantiomeric purity of azelastine enantiomers or the azelastine metabolism. This work can be seen as the basis for future studies applying improved detection systems.

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